PCT/AU99/00434

AU99/434

09/701926

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Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method for modulating plant physiological processes and genetic sequences useful for same"

The invention is described in the following statement:

A METHOD FOR MODULATING PLANT PHYSIOLOGICAL PROCESSES AND GENETIC SEQUENCES USEFUL FOR SAME

FIELD OF THE INVENTION

The present invention relates generally to a method for modulating plant physiological processes such as but not limited to resistance to plant pathogens, senescence, cell growth and the shape of cells, tissues and organs. The method of the present invention is predicated in part on the manipulation of starch metabolism as a means for example, of inducing resistance to plant pathogens and to modulate senescence. In a particular embodiment, the present invention contemplates a method of modulating plant physiological processes by manipulating amylase production in plant cells.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Genetic engineering is now an integral part of strategies to develop varieties of plants with commercially useful traits. Transposons have played an important part in the genetic engineering of plants to provide *inter alia* tagged regions of plant genomes to facilitate the isolation of genes by recombinant DNA techniques as well as to identify important regions in plant genomes responsible for certain physiological processes.

The maize transposon *Activator (Ac)* and its derivative *Dissociation (Ds)* comprise one of the first transposon systems to be discovered (1,2) and was first used to clone genes by Fedoroff *et al* (3). The behaviour of *Ac* in maize has been studied extensively and excision occurs in both somatic and germline tissue. Studies have highlighted two important features of *Ac/Ds* for tagging. First, the transposition frequency and second, the preference of *Ac/Ds* for transposition in linked sites.

The use of the *Ac/Ds* system has been hampered by the difficulty of data interpretation due, for example, to the high activity of *Ac* in certain plants and insertions at unlinked sites arising from multiple transpositions rather than by a single event from the T-DNA. This problem was addressed by Jones *et al* (4), Carroll *et al* (5) and others where a two component *Ac/Ds* system was developed. In this system, the *Ds* elements were made by replacing the *Ac* transposase gene with a marker gene thereby rendering it non-autonomous. T-DNA regions of binary vectors were constructed by Carroll *et al* (5) and Scofield *et al* (6) carrying either a *Ds* element or a stabilised Activator transposase gene *(sAc)*. The *Ds* element contained a reporter gene (eg. *nos:BAR)* which was shown to be inactivated on crossing with plants carrying the *sAc* (5). This is referred to as transgene silencing. It has been shown that transgene silencing is a more general phenomenon in transgenic plants (7, 8, 9). Many different types of transgene silencing have now been reported in the literature and include: co-suppression of a transgene and a homologous endogenous plant gene (10), inactivation of ectopically located homologous transgenes in transgenic plants (7), the silencing of transgenes leading to resistance to virus infection (11) and inactivation of transgenes inserted in maize transposons in transgenic tomato (5).

Gene silencing undoubtedly reflects mechanisms of great importance in the understanding of plant gene regulation. Other important mechanisms include anti-methylation sequences (see Australian Patent Application filed on 4 June 1998 entitled "Expression Modulating Sequences") and negative regulatory sequences (see Australian Patent Application filed on 4 June 1998 entitled "Expression Modulating Sequences-II").

In work leading up to the present invention, the inventors identified yet a further regulatory mechanism involved in controlling plant physiological processes. The mechanism involves modulating starch metabolism and this in turn influences such phenomena as disease resistance, senescence, cell growth and the shape of cells, tissues and organs.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography. A summary of SEQ ID NOs: is given in Table 1.

One aspect of the present invention contemplates a method for controlling physiological processes in a plant said method comprising modulating starch metabolism in cells of said plant.

More particularly, the present invention is directed to a method of inducing a physiological response in a plant said method comprising enhancing or facilitating starch metabolism in cells of said plant after the initial development stage.

Another aspect of the present invention provides a method of inducing a physiological response in a plant such as but not limited to inducing resistance to a plant pathogen, enhancing or delaying senescence, modifying cell growth or altering the shape of cells, tissues or organs, said method comprising modulating synthesis of an amylase or functional derivative thereof for a time and under conditions sufficient for starch metabolism to be facilitated or inhibited.

Still another aspect of the present invention relates to a transgenic plant or a genetically modified plant exhibiting one or more of the following properties:

(i) a non-developmentally silenced amylase gene;

(ii) an amylase gene capable of constitutive or inducible expression;

(iii) a mutation preventing silencing of an amylase gene;

(iv) a nucleic acid molecule proximal to an amylase gene and which substantially prevents

methylation of said amylase gene; and/or

(v) decreased amylase gene expression.

TABLE 1

SUMMARY OF SEQ ID NOs.

SEQ ID NO.	DESCRIPTION
1	Nucleotide sequence of tomato α-amylase gene promoter
2	Nucleotide sequence of potato α-amylase gene promoter
3	Nucleotide sequence of genomic DNA upstream of *Dem* gene followed by *Dem* cDNA coding sequence
4	Nucleotide sequence of putative *Dem* promoter

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation showing T-DNA regions of binary vectors carrying a *Ds* element (SLJ1561) of the transposable gene (SLJ10512)[5]. The *Ds* element carries a *nos:BAR* gene and is inserted into a *nos:SPEC* excision marker. The transposon gene *sAc* is linked to a *2':Gus* reporter gene.

Figure 2 is a diagrammatic representation showing an experimental strategy for generating tomato lines carrying transposed *Ds* elements (5). F1 plants heterozygous for both the *Ds* and *sAc* T-DNAs are test-crossed to produce TC_1 progeny. The TC_1 progeny are then screened for lines carrying a transposed *Ds* and a reactivated *nos:BAR* gene.

Figure 3 is a representation of a sequence comparison between the potato α-amylase promoter [SEQ ID NO:2] (14) and the tomato α-amylase promoter [SEQ ID NO:1]. The location of the UQ406 insertion is shown in bold.

Figure 4 is a diagrammatic representation showing the chromosomal region of the tomato α-amylase, *Dem* and γ genes. The α-amylase and γ coding sequences are shown as shaded boxes and the *Dem* gene as an open box on the chromosome. The region of homology to the potato α-amylase promoter and coding sequence are shown on the figure.

Figure 5 is a photographic representation showing tissue and *in situ* distribution of *Dem* mRNA. a, Northern blot analysis of *Dem* expression in light-grown seedlings (LS), dark-grown seedlings (DS), shoot apices (SA), mature leaves (ML), young fruit (YF), roots (R), stem (S) and callus (C). b-d, *in situ* hybridization with a *Dem* antisense probe. b, shoot apical meristem of a 4 week-old plant. c, dormant auxiliary meristem. d, root apex.

Figure 6 is a photographic representation showing somatic tagging of the *Dem* locus. *a,* leaf showing the somatic tagging of the *Dem* locus. Light coloured sectors on the adaxial side of the leaf represent independent insertions of *Ds* in *Dem.* The appearance of the abaxial side of the leaf is the same as wild-type. *b,* Scanning Electron Microscope (SEM)of a somatic sector

showing abnormal and wild-type epidermal cells. The SEM shows a wild-type sector in the lower right hand half of the figure, and a mutant sector in the upper left hand side. Note that the epidermal and hair cells are larger on the wild-type sector.

Figure 7 is a representation showing that the *Dem* gene is required for palisade cell expansion in the leaf. Transverse sections of (**a**) variegated and (**b**) wild-type leaves. **p** and **s** indicate a palisade cell and spongy mesophyll cell layers, respectively. Light green parts are indicated by **lg**, and green parts by **g**. Light green sectors lacking palisade cells are mutated by *Ds* insertion in the *Dem* gene.

Figure 8 shows PCR on intact tissue of *dem* sectors. **M**, 1 kb ladder. **1-10**, unique *Ds* insertions in *Dem* detected by PCR. Intact leaf tissues (mutant somatic sectors) were used as template in the PCR. PCR with oligonucleotide primers facing out of *Ds* and in the *Dem* coding sequence amplified unique fragments from each mutant sector, thereby confirming that the sectors shown in Figures 6 and 7 are indeed mutant *dem* sectors.

Figure 9 is a diagrammatic representation showing an improved transposon tagging strategy using *Dem* as excision marker. The *sAc* and *Ds* parent lines are represented by the upper left and right boxes, respectively. Because the stabilised *sAc* is linked to the frameshift *dem* allele in one parent, somatic revertants occur at the frequency of about 1 out of 4 in the F1 progeny. Each somatic revertant represents an independent transposition event. Chr4, chromosome 4 of tomato.

Figure 10 is a representation of the nucleotide sequence [SEQ ID NO:3] of genomic DNA from 651 bp upstream of the *Ds* insertion in UQ406 to the beginning of the *Dem* coding sequence, followed by the *Dem* cDNA sequence from the ATG start site at base pair 4097. The target sequences of UQ406 and *Dem* ATG are underlined. The *Dem* cDNA sequence is shown in italics and is underlined. The putative *Dem* promoter is 709 bases long beginning at nucleotide 3388 and ending just prior to the ATG, i.e. at position 4096 [SEQ ID NO:4].

Figure 11 is a photographic representation showing the dominant lesion mimic phenotype of

UQ406. The leaf tissue on the left is wild-type, on the right is UQ406. Young and old leaves are shown in the upper and lower portions of the figure, respectively. No symptoms have been observed on young differentiating tissue of UQ406.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, transposon-mediated tagging of tomato plants was shown to result in the identification of mutants exhibiting altered physiological properties. In particular, the insertion of a transposon in close proximity to the α-amylase gene resulted in continued or modified expression of the α-amylase gene past the initial development stage of the plant. In wild-type plants, negative regulatory mechanisms which may include methylation result in the non-expression of the α-amylase gene. In accordance with the present invention, modified expression of the α-amylase gene, post or after initial developmental stage, results in physiological attributes such as altered senescence, altered resistance to pathogens, modification of the shape of plant cells, tissues and organs and altered cell growth characteristics. It is proposed, in accordance with the present invention, that the altered physiological phenotype is due to modified starch metabolism by the continued or modified expression of the α-amylase gene. In particular, increased or modified expression of the α-amylase gene or otherwise continued or altered expression of the α-amylase gene post initial development results in cell death, i.e. cell apoptosis, but also induces or promotes resistance to pathogens.

Accordingly, one aspect of the present invention contemplates a method for controlling physiological processes in a plant said method comprising modulating starch metabolism in cells of said plant.

More particularly, the present invention is directed to a method of inducing a physiological response in a plant said method comprising inhibiting or facilitating starch metabolism in cells of said plant after the initial developmental stage.

The present invention is exemplified herein with respect to the effects of starch metabolism in tomato plants. This is done, however, with the understanding that the present invention extends to the manipulation of starch metabolism in any plant such as flowering plants, crop plants, ornamental plants, vegetable plants, native Australian plants as well as Australian and non-Australian trees, shrubs and bushes.

Physiological responses contemplated by the present invention include but are not limited to cell apoptosis, senescence, pathogen resistance, cell, tissue and organ shape and plant growth.

In a particularly preferred embodiment, starch metabolism is stimulated, promoted or otherwise enhanced or inhibited by manipulating levels of an amylase and this in turn may lead to *inter alia* senescence or apoptosis as well as resistance to pathogens. Reference to "amylase" includes any amylase associated with starch metabolism including α-amylase and β-amylase. This aspect of the present invention also includes mutant amylases. In addition, the manipulation of levels of amylase may be by modulating endogenous levels of a target plant's own amylase, or an exogenous amylase gene or antisense, co-suppression or ribozyme construct may be introduced into a plant. The exogenous amylase gene may be from another species or variety of plant or from the same species or variety or from the same plant. The present invention extends to recombinant amylases and derivative amylases including fusion molecules, hybrid molecules and amylases with altered substrate specifications and/or altered regulation.

According to another aspect of the present invention there is provided a method of inducing a physiological response in a plant such as but not limited to inducing resistance to a plant pathogen, enhancing or delaying senescence, modifying cell growth or altering the shape of cells, tissues or organs, said method comprising modulating synthesis of an amylase or functional derivative thereof for a time and under conditions sufficient for starch metabolism to be modified.

Preferably, the amylase is α-amylase.

The manipulation of amylase levels may be by manipulating the promoter for the amylase gene, inhibiting or promoting negative regulatory mechanisms such as described in an Australian Patent Application filed on 4 June 1998 entitled "Expression Modulating Sequences - II" or introducing anti-methylation sequences such as those described in an Australian Patent Application filed on 4 June 1998 entitled "Expression Modulating Sequences". Alternatively, an exogenous amylase gene may be introduced or an exogenous promoter designed to enhance expression of the endogenous amylase gene.

The present invention further extends to a transgenic plant or a genetically modified plant exhibiting one or more of the following characteristics:

(i) a non-developmentally silenced amylase gene;

(ii) an amylase gene capable of constitutive or inducible expression;

(iii) a mutation preventing silencing of an amylase gene;

(iv) a nucleic acid molecule proximal to an amylase gene and which substantially prevents methylation of said amylase gene; and/or

(v) decreased amylase gene expression.

The term "proximal" is used in its most general sense to include the position of the amylase gene near, close to or in the genetic vicinity of the nucleic acid molecule referred to in part (iv) above. More particularly, the term "proximal" is taken herein to mean that the amylase gene precedes, follows or is flanked by the nucleic acid molecule. Preferably, the amylase is within the nucleic acid molecule and, hence, is flanked by portions of the nucleic acid molecule. Generally, the amylase gene is flanked by up to about 100 kb either side of the nucleic acid molecule, more preferably up to about 10 kb, even more preferably to about 4 kb either side of the nucleic acid molecule and even more preferably up to about 10 bp to about 1 kb.

Accordingly, another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides which stabilises, increases or enhances expression of an amylase gene inserted into, flanked by, adjacent to or otherwise proximal to the said nucleic acid molecule.

In an alternative embodiment, the present invention contemplates an isolated nucleic acid molecule comprising a sequence of nucleotides which inhibits, decreases or otherwise reduces expression of an amylase gene inserted into, flanked by, adjacent to or otherwise proximal to the said nucleic acid molecule.

The term "expression" is conveniently determined in terms of desired phenotype. Accordingly, the expression of a nucleotide sequence may be determined by a measurable phenotypic change

such as resistance to a plant pathogen, enhanced or delayed senescence, altered cell growth or altered cell, tissue or organ shape.

The nucleic acid molecule described above is referred to herein as an "expression modulating sequence" (EMS) since it functions to and is capable of modulating expression of an amylase gene or its derivatives. The term "modulating" includes increasing or stabilising expression of the amylase gene or decreasing or inhibiting the amylase gene. An EMS may be a co-suppression molecule, ribozyme, antisense molecule, an anti-methylation sequence, a methylation-inducing sequence and/or a negative regulatory sequence, amongst other molecules.

Accordingly, another aspect of the present invention relates to an expression modulating sequence (EMS) comprising a sequence of nucleotides which increases, enhances or stabilizes expression of an amylase gene inserted within, adjacent to or otherwise proximal with said EMS.

In an alternative embodiment, the present invention provides an expression modulating sequence (EMS) comprising a sequence of nucleotides which inhibits, decreases or otherwise reduces expression of an amylase gene inserted within, adjacent to or otherwise proximal with said EMS.

Another aspect of the present invention contemplates a genetic construct comprising an EMS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said EMS wherein said nucleotide sequence encodes an amylase or functional derivative thereof.

The term "genetic construct" is used in its broadest sense to include any recombinant nucleic acid molecule and includes a vector, binary vector, recombinant virus and gene construct.

The means to facilitate insertion of a nucleotide sequence include but are not limited to one or more restriction endonuclease sites, homologous recombination, transposon insertion, random insertion and primer and site-directed insertion mutagenesis. Preferably, however, the means is one or more restriction endonuclease sites. In the case of the latter, the nucleic acid molecule is cleaved and another nucleotide sequence ligated into the cleaved nucleic acid molecule.

Preferably, the amylase gene sequence is operably linked to a promoter in the genetic construct.

According to this embodiment, there is provided a genetic construct comprising an EMS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said EMS and operably linked to a promoter wherein said nucleotide sequence encodes an amylase or functional derivative thereof.

Conveniently, the genetic construct may be a transposable element such as but not limited to a modified form of *Ds*. A modified form of *Ds* includes a *Ds* molecule comprising an EMS and a nucleotide sequence such as but not limited to a reporter gene and a gene encoding an amylase.

Another aspect of the present invention contemplates a method of increasing or stabilising expression of a nucleotide sequence encoding an amylase or otherwise preventing or reducing silencing of a nucleotide sequence encoding an amylase in a plant cell said method comprising introducing into said plant or plant cells said nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with an EMS.

In an alternative embodiment, the present invention provides a method of inhibiting, decreasing or otherwise reducing expression of a nucleotide sequence encoding an amylase in a plant cell said method comprising introducing into said plant or plant cells said nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with an EMS.

Yet another aspect of the present invention provides a transgenic plant carrying a nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with an EMS.

Still a further aspect of the present invention provides nucleic acid molecules encoding apoptopic peptides, polypeptides or proteins or nucleic acid molecules which themselves confer apoptosis. One example of an apoptopic nucleic acid molecule is a molecule capable of inducing or enhancing amylase synthesis. Other molecules are readily identified, for example, by a differential assay. In this example, nucleic acid sequences (e.g. DNA, cDNA, mRNA) are isolated from wild type plants and mutant plants which exhibit enhanced or modified amylase

gene expression. The differential assay seeks to identify DNA or mRNA molecules in the mutant plant or wild type plant which are absent in the respective wild type plant or mutant plant. Such nucleic acid molecules are deemed putative apoptosis-inducing or apoptosis-inhibiting genetic sequences. These molecules may have utility in regulating beneficial physiological processes in plants.

The present invention is further directed to the putative *Dem* promoter and its further derivatives. This is approximately 709 bases in length extending upstream from the ATG start site. The nucleotide positions of putative *Dem* promoter are nucleotide 3388 to 4096 (Figure 10).

The present invention further described by the following non-limiting Examples.

EXAMPLE 1

Ds Transposon tagging of an α-amylase gene affecting plant development

The inventors have previously developed a two component *Ds/sAc* transposon system in transgenic tomato for tagging and cloning important genes from plants (5, 12). The components of the system are shown in Figure 1 and comprise: i) a non-autonomous genetically-engineered *Ds* element (e.g. SLJ1561), and ii) an unlinked transposase gene *sAc* (SLJ10512), required for transposition of the *Ds* element. To activate transposition, the two components are combined by crossing transformants for each component. A plant selectable marker gene, e.g. *nos:BAR,* is inserted into the *Ds* element to enable selection for reinsertion of the elements following excision from the T-DNA (Figure 1). Surprisingly, the marker gene is irreversibly inactivated when the *Ds* line is crossed to a transformant expressing the transposase gene (5). Silencing occurred when the *Ds* element remained in the T-DNA, and also occurred in the great majority of cases when the *Ds* element transposed to a new location in the tomato genome. None of the other marker genes in the T-DNA is silenced. The silenced marker gene has been shown to be stably inherited, even after the transposase gene segregates away from the *Ds* element in subsequent generations.

The experimental strategy for generating tomato lines carrying transposed *Ds* elements from T-DNA 1561E is shown in Figure 2. One line, called UQ406, carries a single transposed *Ds* element (without the transposase gene which has segregated away) and is characterised by showing a disease mimic or premature senescence phenotype on mature leaves. UQ406 also possesses an active *nos:BAR* gene indicating that the insertion caused two phenotypes; namely premature senescence and reactivation of the *nos:BAR* gene inside the *Ds* element.

GenomeWalker (13) is used to clone the tomato DNA sequences flanking the *Ds* element in UQ406. The DNA flanking the *Ds* element in line UQ406 is cloned and sequenced, and a search of the PROSITE database reveals that the *Ds* has inserted into the promoter region of an α-amylase gene. The promoter shows strong homology to an α-amylase promoter of potato (14; Figure 3) and the coding sequence of the gene has strong homology with one of 3 reported potato α-amylase cDNAs (15). Surprisingly, DNA sequence analysis also shows that the *Ds*

insertion in UQ406 is located only about 3 kb upstream from the ATG of the *Dem* (Defective embryo and meristems) gene which has been cloned by tagging with *Ds*. In fact, only about 700 bp of DNA separates the putative α-amylase STOP codon and the *Dem* ATG codon (Figure 4). The *Dem* gene is required for correct patterning in all of the major sites of differentiation, namely in the embryo, meristems, and organ primordia (Figure 5). The inventors have shown by somatically tagging *Dem* with *Ds*, that the gene is involved in cell expansion during plant differentiation (Figures 6, 7 and 8). The close proximity of the α-amylase and *Dem* genes indicates that the α-amylase gene may also be involved in cell expansion during plant differentiation. The sequence flanking the active *nos:BAR* genes are referred to herein as "Expression Modulating Sequences" or "EMSs".

EXAMPLE 2

An improved transposon tagging strategy for transgenic tomato

The inventors have used the transposon tagging system described in Example 1 (also see Figure 2) to tag and clone three important genes involved in shoot morphogenesis: the *DCL* gene, required for chloroplast development and palisade cell morphogenesis (12); the *Dem* gene, required for cotyledon development and shoot meristem function; and the α-amylase gene, described in Example 1 above.

Stable *Ds* insertion mutants of *Dem* germinate but fail to develop any further. However, variegated seedlings appear at first to be mutant, but the transposase gene activates transposition of the *Ds* and reversion of the *Dem* locus to wild-type, thereby restoring function to the shoot meristem. While the transposon tagging system described in Figure 2 has been successful in tagging genes and chromosomal regions alleviating transgene silencing, it does have two associated inefficiencies. First, transposition cannot be selected in the shoot meristem of F_1 plants heterozygous for *Ds* and *sAc*. As a consequence, many TC_1 progeny derived from test-crossing these F_1 plants still have the *Ds* located in the T-DNA. The other limitation of the system is that sibling TC_1 progeny derived from a single F_1 plant often carry the same clonal transposition and reinsertion event. The extent of clonal events amongst sibling TC_1 progeny can only be monitored by time consuming and expensive Southern hybridization.

These two inefficiencies in the transposon tagging strategy are overcome in accordance with the present invention by using the *Dem* gene as an excision marker. The new system enables selection for transposition in the shoot apical meristem and visual identification of plants carrying independent transposition events. Transposition is initiated by crossing a *Ds* line with a *sAc* line (Figure 9). The *Ds* line is heterozygous for a *Ds* insertion in the *Dem* gene and the *sAc* line is heterozygous for a stable frameshift mutation in the *Dem* gene (Figure 9). The frameshift allele is derived from a *Ds* excision event from the *Dem* locus. Both the *Ds* and *sAc* lines are wild-type due to the recessive nature of the *Ds* insertion and frameshift alleles. PCR tests on intact leaf tissue have been developed for the rapid identification of these *Ds* and *sAc* parental lines. The F_1 progeny derived from crossing the *Ds* and *sAc* lines segregate at the expected ratio of 3 wild-types to 1 mutant. Because the stabilised *sAc* is linked to the frameshift *dem* allele almost all of the F_1 mutants also inherit the transposase gene *(sAc)* and can undergo somatic reversion. These revertant individuals have abnormal cotyledons, but *Ds* excision from the *Dem* gene restores function to the shoot apical meristem. Each somatic revertant represents an independent transposition event from the *Dem* locus. A non-destructive test for *nos:BAR* expression is used involving application of PPT (the selective agent for expression of *BAR* gene) to a small area of a leaf. Somatic revertants resistant to PPT are grown though to seed and the F_2 progeny are screened again for PPT resistance. Lines carrying transposed *Ds* elements are selected for more detailed molecular analysis. Independent *Ds* insertions in the vicinity of *Dem* and the α-amylase gene are identified by PCR.

EXAMPLE 3

Modification of plant cell, tissues and organ shapes and plant growth by genetic manipulation of α-amylase

The DNA from 651 bp of the upstream of the UQ406 insertion down to the end of the *Dem* coding sequence has been sequenced (Figure 10). The close proximity of the α-amylase gene to the *Dem* cell expansion gene indicates that these genes may play a key role in cell expansion and differentiation. Several heterozygous insertion mutants are identified in the α-amylase coding sequence and these are selfed to produce plants homozygous for the *Ds* insertion in the α-

amylase coding sequence. If these have a similar or more or less severe phenotype to the plants homozygous for the stable *Dem* insertion mutant, then this will indicate that indeed this cloned α-amylase gene plays a key role in cell expansion, and, therefore, the shape and growth of plants. Several heterozygous insertion mutants have been identified in the γ coding sequence downstream of the *Dem* coding sequence (Figure 4) and these are selfed to produce plants homozygous for the *Ds* insertion in the γ coding sequence. If these have a similar or more or less severe phenotype to the plants homozygous for the stable *Dem* insertion mutant, then this will indicate that the γ gene also has a role in cell expansion and the shape and growth of plants.

A tomato chromosomal region spanning these genes is cloned into an *Agrobacterium* binary vector (16) to produce plasmid pUQ113, and this plasmid is introduced into *Arabidopsis* by method of (17) to modify the cell shape and growth of this other plant species. A T-DNA insertion mutant in the *Dem* gene is identified in *Arabidopsis* and this mutant is also transformed with pUQ113 to modify the cell shape and growth of *Arabidopsis*.

Recombinant combinations of α-amylase and *Dem* genes are transformed into a range of plant species to modify the cell shape and growth of the species.

EXAMPLE 4

Genetic engineering of disease resistance and senescence based on modification of expression of α-amylase

Ds insertion mutant UQ406 is characterized by a lesion mimic phenotype. The mutant phenotype is evident in mature leaves (Figure 11), but not in young leaves or any other tissue. No pathogens are found in leaf tissue displaying this phenotype. The dominant nature of the UQ406 phenotype and the location of the *Ds* in the α-amylase promoter suggest that over-, under or constitutive expression of the gene may be responsible for activating a disease resistance response and/or senescence in mature leaves. These data and the very close proximity of the α-amylase and *Dem* genes are also consistent with co-ordinate regulation of these genes in differentiating tissue. Induction of disease resistance and plant senescence, to produce desirable outcomes in crops and

plant products, may, therefore, be able to be controlled by modification of α-amylase expression.

An early event in the disease response of a challenged plant is a major respiratory burst, often referred to as an oxidative burst due to an increase in oxygen consumption. This burst of oxygen consumption is due to the production of hydrogen peroxide (H_2O_2) linked to a surge in hexose monophosphate shunt activity (19). This activity results from the activation of a membrane-bound NADPH oxidase system which catalyses the single electron reduction of oxygen to form superoxide (HO_2/O_2^-), using NADPH as the reductant (19). Spontaneous dismutation of HO_2/O_2^- then yields $H_2 O_2$. Consumption of glucose *via* the hexose monophosphate shunt (alternatively known as the cytosolic oxidative pentose phosphate pathway) regenerates the NADPH consumed by the NADPH oxidase system. It is, therefore, entirely conceivable that an α-amylase is responsible for supplying sugars required by the pentose phosphate pathway, and perhaps for the primary activation of the signal transduction pathway that leads to disease resistance in plants.

Following the oxidative burst, disease resistance is manifested in localised plant cell death called the hypersensitive response (HR), in the vicinity of the pathogen. The HR may then induce a form of long-lasting, broad spectrum, systemic and commercially important resistance known as systemic acquired resistance (SAR). The compounds, salicylic acid, jasmonic acid and their methyl derivatives as well as a group of proteins known as pathogenesis related (PR) proteins are used as indicators of the induction of SAR (18).

Increased levels of sugars have been related to heightened resistance especially to biotrophic pathogens (20). When invertase (the enzyme responsible for the breakdown of sucrose to glucose and fructose) is overexpressed in transgenic tobacco, systemic acquired resistance is induced (21).

The α-amylase coding sequence is inserted behind an inducible promoter and transformed into plants to confer a inducible disease resistance in plants. Similarly, the α-amylase coding sequence is inserted behind an inducible promoter and transformed into plants to confer inducible senescence in plants for the production of desirable products or traits.

When a disease resistance response is invoked in one part of a plant, a general and systemic acquired enhancement in disease resistance is conferred on all tissues of such a plant (18). Tomato line UQ406 is tested for enhanced resistance to a wide range of pathogens to test this hypothesis.

EXAMPLE 5

Cloning of downstream genes associated with plant cell apoptosis caused by *Ds* insertion

A cDNA library is made from tomato leaf tissue showing the disease mimic (apoptosis) phenotype caused by *Ds* insertion. This library is screened differentially with two probes, one being cDNA from normal tissue and the other being cDNA made from leaf tissue showing the disease mimic phenotype caused by *Ds* insertion. This procedures identifies genes specifically-induced during plant cell death. These apoptosis-associated genes are then sequenced, and compared with other genes present in the DNA databases. The proteins encoded by these genes are expressed *in vitro* and tested for their ability to kill plant cells.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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15. International Patent Publication No. WO 90/12876-A.
16. Dixon, M.S. *et al.* (1996) *Cell 84:* 451-459.
17. Bechtold, N. and Bouchez, D. (1995) In: I. Potrykus and G. Spangenberg (eds). Gene transfer to Plants. pp.19-23.
18. Ryals, J. *et al.* (1995). Proceedings of the National Academy of Sciences of the United States of America, 92: 4202-4205.
19. Pugin, A. *et al.* (1997) *Plant Cell 9:* 2077-2091.
20. Vanderplank, J.E. (1984) "Sink-induced loss of resistance". In *Disease resistance in plants* (2nd Ed.), J. E. Vanderplank, ed. (London: Academic Press), pp. 107-116.
21. Herbers, K., Meuwly, P., Frommer, W.B., Metraux, J-P., and Sonnewald, U. (1996). *The Plant Cell 8:* 793-803.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE UNIVERSITY OF QUEENSLAND

(ii) TITLE OF INVENTION: A METHOD FOR MODULATING PLANT PHYSIOLOGICAL PROCESSES AND GENETIC SEQUENCES USEFUL FOR SAME

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: AUSTRALIAN PROVISIONAL
(B) FILING DATE: 4-JUN-1998
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES, DR E JOHN L
(C) REFERENCE/DOCKET NUMBER: EJH/AF

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770
(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1217 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCTTACTGTT GTGCTCAAAG CAACTTCATC ATCATACAGT ATGGTTTTGA TATGCTCTTC 120

CATTATCACT GAGCCTTATG ATTATGTTTT ACGAGCTTAT AATATCACTG ATGGTGATTC 180

AGTATTGTGA TTATGTCCTT CGTTGATTAT TCTGTTTCAT ACAAGTCGTG TAATTTGCTG 240

TTTGTGACAG TACGATAGAT CGACTCAACC TTCTGAGGTA TTAGTTGAAG TTCATGTAAA 300

TTAGCTTTGT TTATCATAGT AGCATTTGAT TATTGATGCT CTGTAGCTAA TGATAAGCCA 360

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TTCATCTAGC CCACAACCGT GGTGGAGGAT CTAGAATTTT CATGAAAGGA TTCAAAATTT 720

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CACAACTCCC GTGTCTTGTG TGCTCGTCGC TCAGCATGCA AGTCGAGAAA AGAAAGACCA 900

AAACAATGAA AACTTTACGA AAAATCAAAA AGTTGAAGGA CTTTAACGTC GAGATCTCTC 960

GTAGAAAACC TCTTTTGTAA GGTTGCATAC AATACTTTTT TTTCAGACTT TACTTATGGT 1020

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1114 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTATGATTAT GTCCTCCATT AATTATTCTG TTTCATACAA GTCGTGTAAT TTGCTGTTTG 240

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CAGTTTTTTA GTCTCTAAAA CACATTTAAA TAGAGTTTAT TTGCCATCTT TTGTTCCTCA 1080

TACTAGACTT CGGAGTCAAC ACAACACAAC AACA 1114

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6263 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AACTTCATCA TCATACAGTA TGGTTTTGAT ATGCTCTTCC ATTATCACTG AGCCTTATGA 180

TTATGTTTTA CGAGCTTATA ATATCACTGA TGGTGATTCA GTATTGTGAT TATGTCCTTC 240

GTTGATTATT CTGTTTCATA CAAGTCGTGT AATTTGCTGT TTGTGACAGT ACGATAGATC 300

GACTCAACCT TCTGAGGTAT TAGTTGAAGT TCATGTAAAT TAGCTTTGTT TATCATAGTA 360

GCATTTGATT ATTGATGCTC TGTAGCTAAT GATAAGCCAT TGGAGGGAAG CAAGCTTTCT 420

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 708 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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ACATATGTTG AAATTACCCT TTATCCCCTT ACAATTGATT TAATAAATAT TTCCCCTATC 240

CCTTTGGTAG TTGGTTAGAG TTATAAGTAA CGTAGAGATT AGTTATAAGA GAATTTATGT 300

ATTATTATGC AGATGTTTAG TTATATCGAT TTTAGTTATT TATATGTTGA TTATTTCACC 360

TTCAATAATG CATATAAAGA TGGTAAATGA TTGGATTGAT CGAATTCGAA TGAGTTTGAA 420

TATGAACTAA TCTTCAAATT TAATATAAAT TTTTTTTGTC AACATCTATA GCCAAACGGC 480

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TTTGAAAATT GCGCTCCTCA CAGTTCTCCC CTATTCAGAT TTGATTCATT CTCTTCATTT 660

TTTGTTTTCA CATTTTACCT CTAAATCAAC TCGAGTCCCT TTGTTCAA 708

DATED this 4th day of June 1998

THE UNIVERSITY OF QUEENSLAND

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

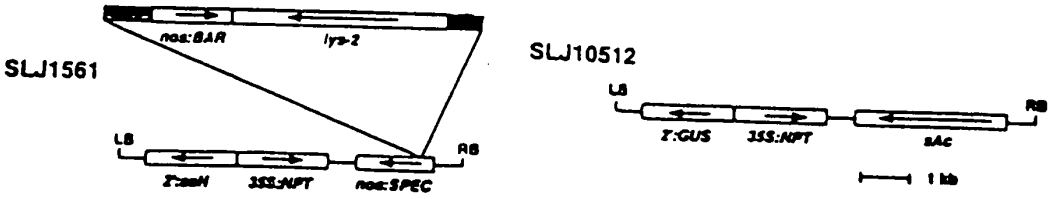
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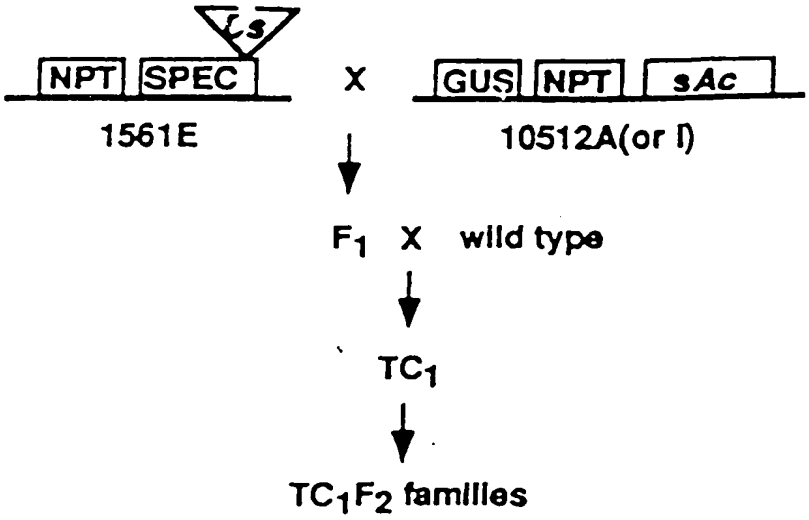
FIGURE 1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TC1
TC1F2 families

FIGURE 2

FIGURE 3 (i)

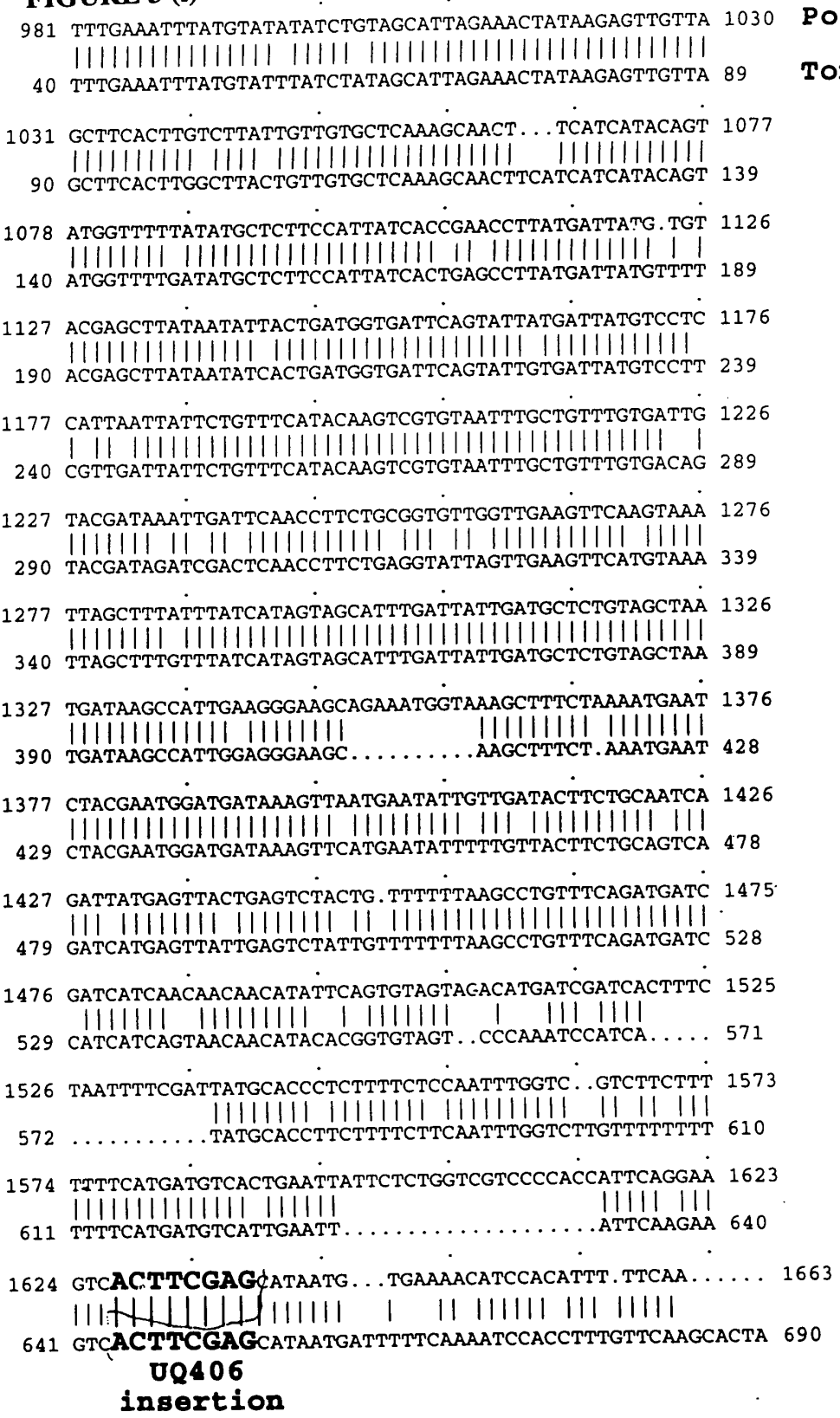
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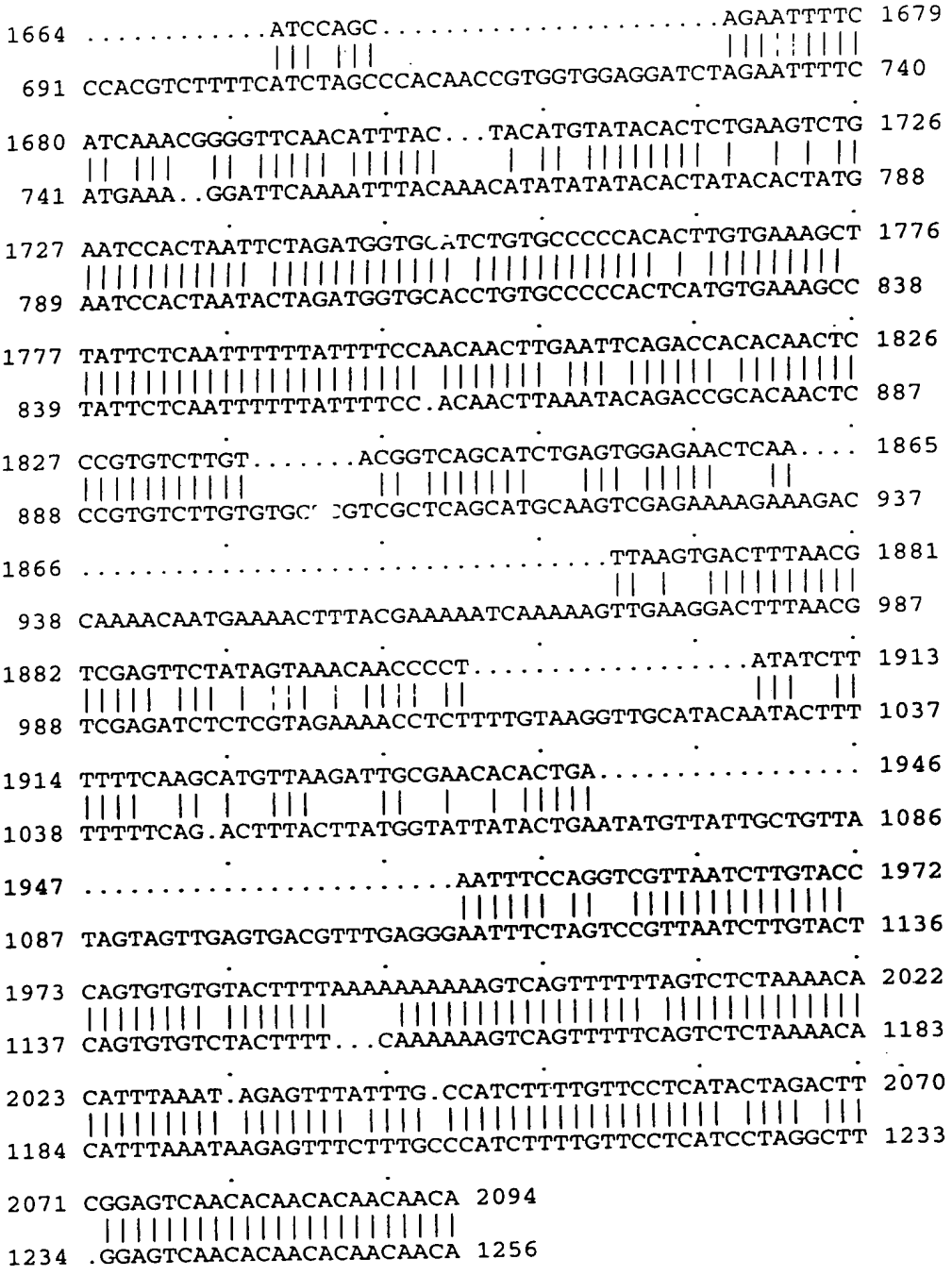
FIGURE 3 (ii)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988 TCGAGATCTCTCGTAGAAAACCTCTTTTGTAAGGTTGCATACAATACTTT 1037
1914 TTTTCAAGCATGTTAAGATTGCGAACACACTGA..... 1946
1038 TTTTTCAG.ACTTTACTTATGGTATTATACTGAATATGTTATTGCTGTTA 1086
1947 .....AATTTCCAGGTCGTTAATCTTGTACC 1972
1087 TAGTAGTTGAGTGACGTTTGAGGGAATTTCTAGTCCGTTAATCTTGTACT 1136
1973 CAGTGTGTGTACTTTTAAAAAAAAAAGTCAGTTTTTTAGTCTCTAAAACA 2022
1137 CAGTGTGTCTACTTTT...CAAAAAAGTCAGTTTTTCAGTCTCTAAAACA 1183
2023 CATTTAAAT.AGAGTTTATTTG.CCATCTTTTGTTCCTCATACTAGACTT 2070
1184 CATTTAAATAAGAGTTTCTTTGCCCATCTTTTGTTCCTCATCCTAGGCTT 1233
2071 CGGAGTCAACACAACACAACAACA 2094
1234 .GGAGTCAACACAACACAACAACA 1256

FIGURE 4

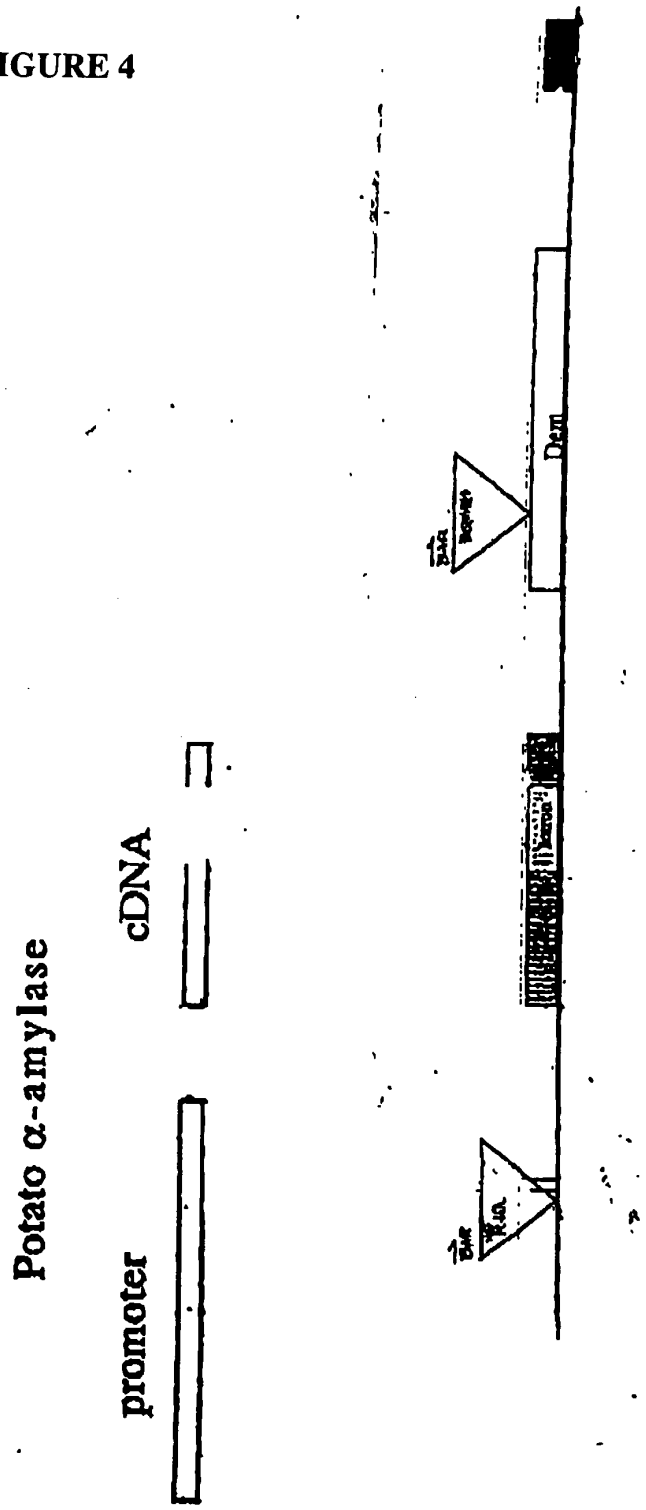


FIGURE 5

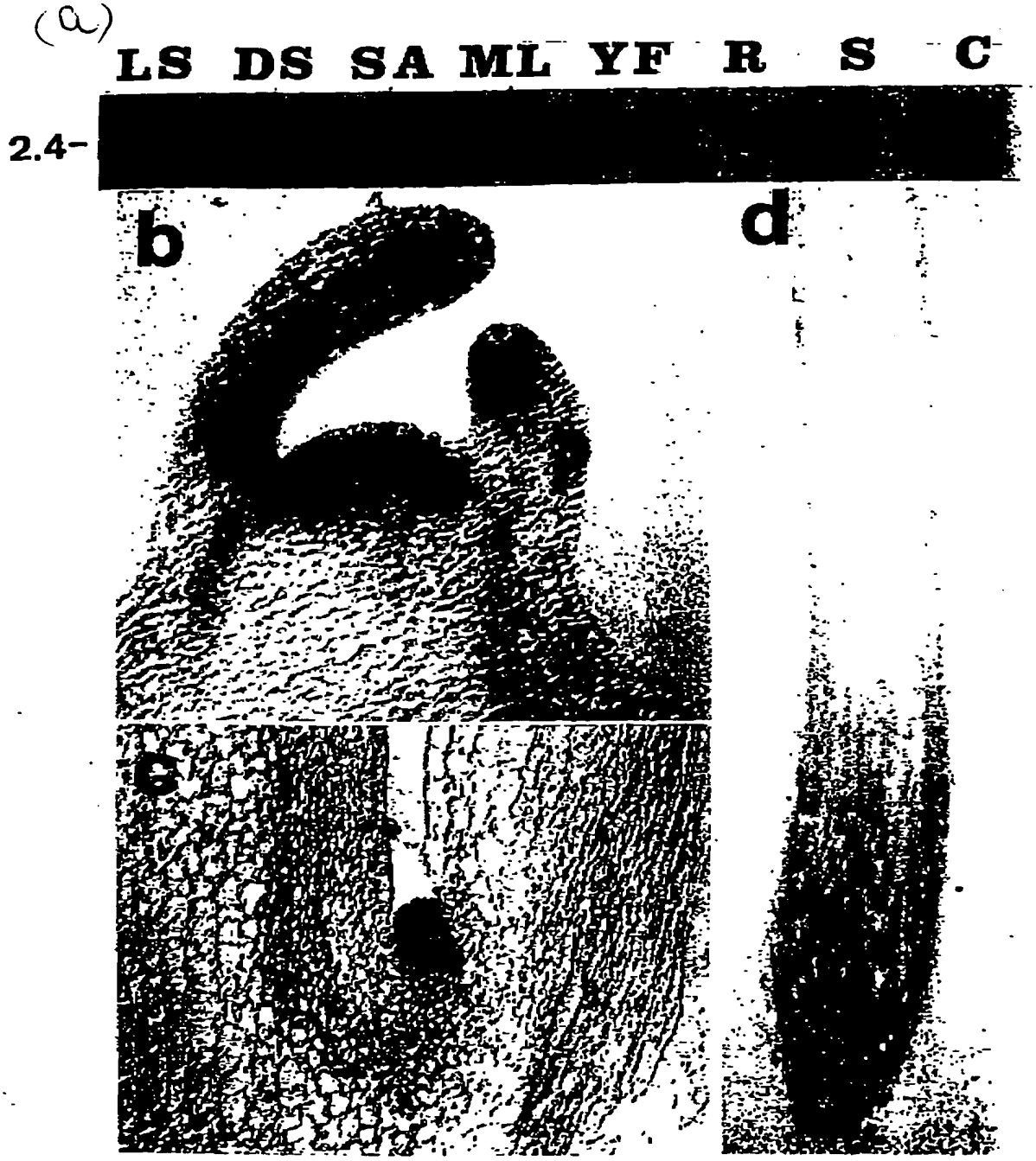
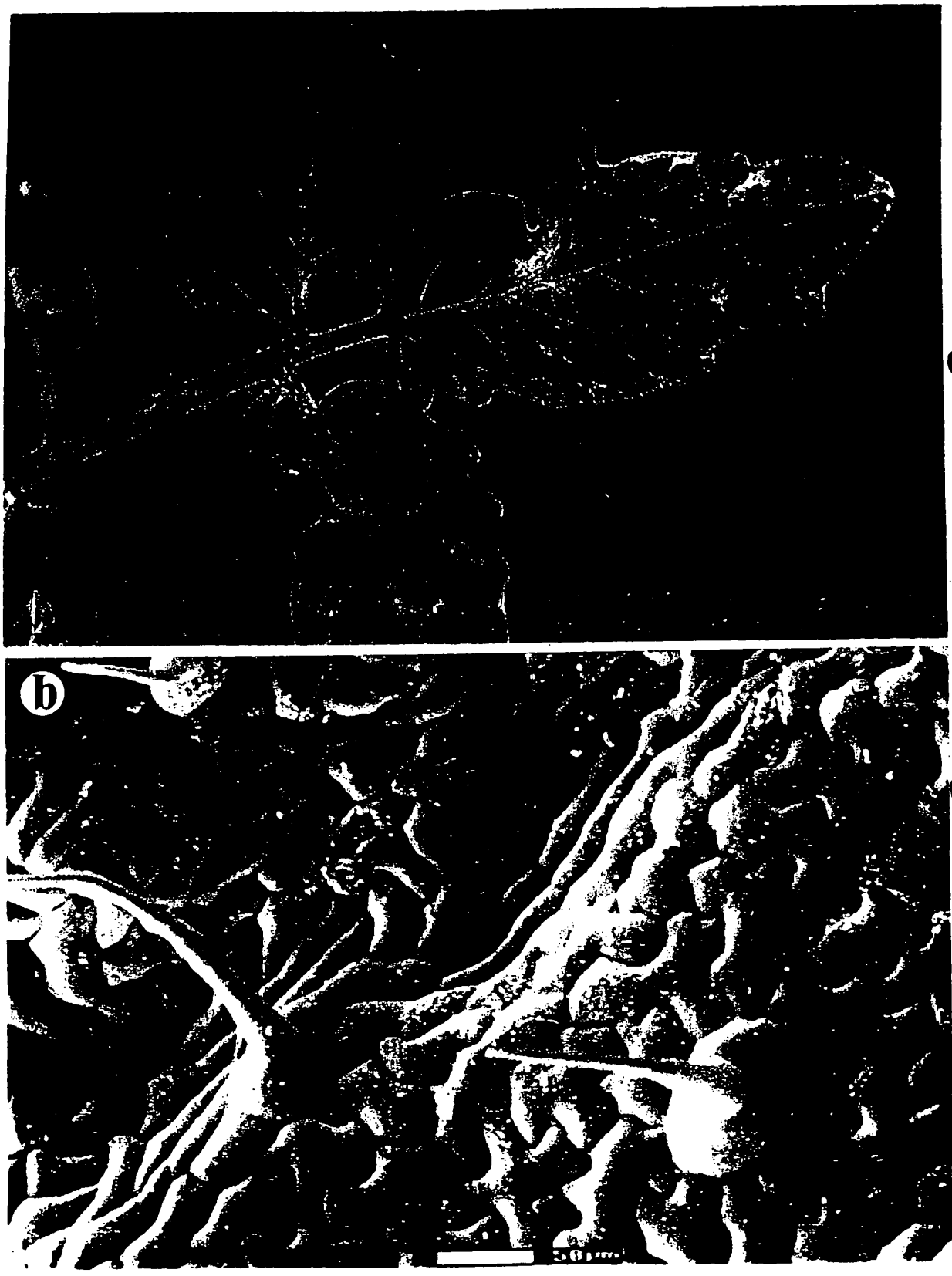


FIGURE 6



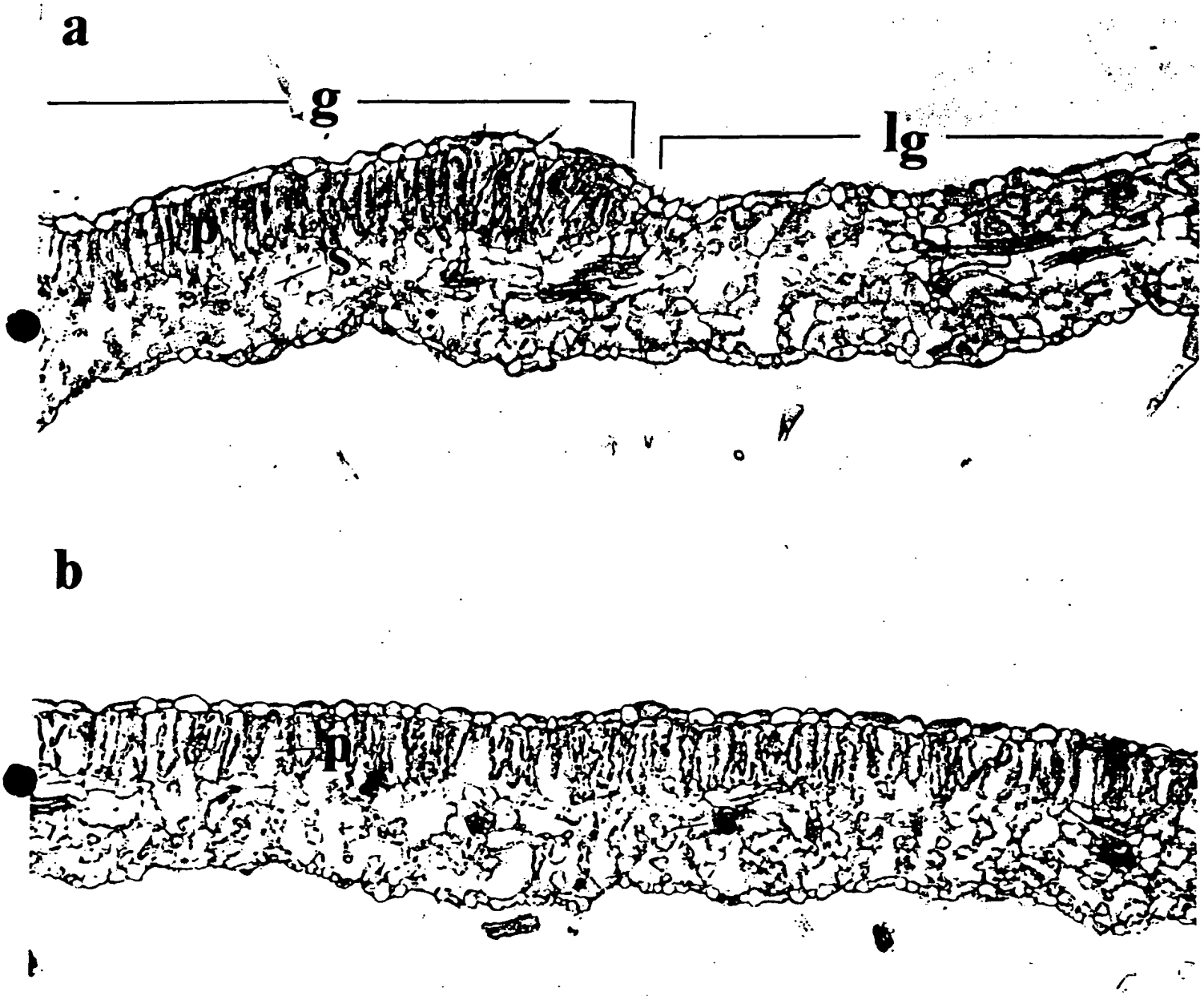
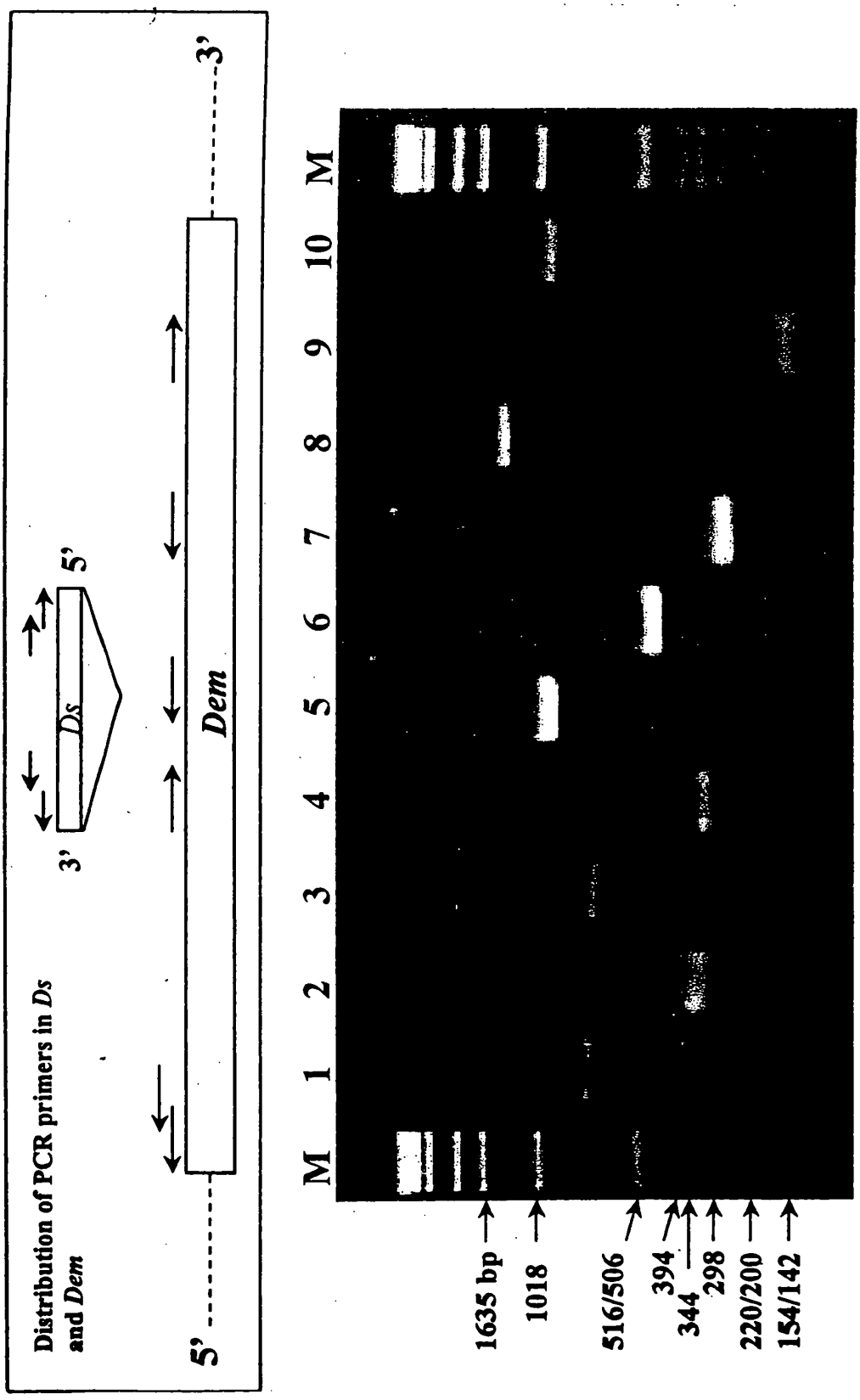


FIGURE 7

FIGURE 8


Distribution of PCR primers in Ds and Dem
5'
3'
Ds
3'
5'
Dem
M 1 2 3 4 5 6 7 8 9 10 M
1635 bp
1018
516/506
394
344
298
220/200
154/142

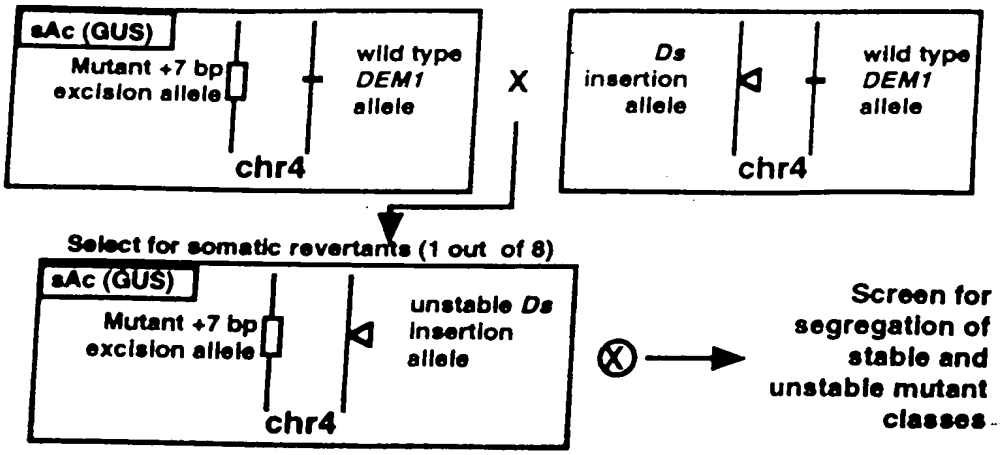

sAc (GUS)
Mutant +7 bp excision allele
wild type DEM1 allele
chr4
X
Ds insertion allele
wild type DEM1 allele
chr4
Select for somatic revertants (1 out of 8)
sAc (GUS)
Mutant +7 bp excision allele
unstable Ds insertion allele
chr4
Screen for segregation of stable and unstable mutant classes

FIGURE 9

FIGURE 10 (i)

```
   1 CGACGGCCCG GGCTGGTAAA TGCGGAAGCT TGTTACAGAT TTGAAATTTA
  51 TGTATTTATC TATAGCATTA GAAACTATAA GAGTTGTTAG CTTCACTTGG
 101 CTTACTGTTG TGCTCAAAGC AACTTCATCA TCATACAGTA TGGTTTTGAT
 151 ATGCTCTTCC ATTATCACTG AGCCTTATGA TTATGTTTTA CGAGCTTATA
 201 ATATCACTGA TGGTGATTCA GTATTGTGAT TATGTCCTTC GTTGATTATT
 251 CTGTTTCATA CAAGTCGTGT AATTTGCTGT TTGTGACAGT ACGATAGATC
 301 GACTCAACCT TCTGAGGTAT TAGTTGAAGT TCATGTAAAT TAGCTTTGTT
 351 TATCATAGTA GCATTTGATT ATTGATGCTC TGTAGCTAAT GATAAGCCAT
 401 TGGAGGGAAG CAAGCTTTCT AAATGAATCT ACGAATGGAT GATAAAGTTC
 451 ATGAATATTT TTGTTACTTC TGCAGTCAGA TCATGAGTTA TTGAGTCTAT
 501 TGTTTTTTTA AGCCTGTTTC AGATGATCCA TCATCAGTAA CAACATACAC
 551 GGTGTAGTCC CAAATCCATC ATATGCACCT TCTTTTCTTC AATTTGGTCT
 601 TGTTTTTTTT TTTTCATGAT GTCATTGAAT TATTCAAGAA GTCACTTCGA
 651 GCATAATGAT TTTTCAAAAT CCACCTTTGT TCAAGCACTA CCACGTCTTT
 701 TCATCTAGCC CACAACCGTG GTGGAGGATC TAGAATTTTC ATGAAAGGAT
 751 TCAAAATTTA CAAACATATA TATACACTAT ACACTATGAA TCCACTAATA
 801 CTAGATGGTG CACCTGTGCC CCCACTCATG TGAAAGCCTA TTCTCAATTT
 851 TTTATTTTCC ACAACTTAAA TACAGACCGC ACAACTCCCG TGTCTTGTGT
 901 GCTCGTCGCT CAGCATGCAA GTCGAGAAAA GAAAGACCAA AACAATGAAA
 951 ACTTTACGAA AAATCAAAAA GTTGAAGGAC TTTAACGTCG AGATCTCTCG
1001 TAGAAAACCT CTTTTGTAAG GTTGCATACA ATACTTTTTT TTCAGACTTT
1051 ACTTATGGTA TTATACTGAA TATGTTATTG CTGTTATAGT AGTTGAGTGA
1101 CGTTTGAGGG AATTTCTAGT CCGTTAATCT TGTACTCAGT GTGTCTACTT
1151 TTCAAAAAAG TCAGTTTTTC AGTCTCTAAA ACACATTTAA ATAAGAGTTT
1201 CTTTGCCCAT CTTTTGTTCC TCATCCTAGG CTTGGAGTCA ACACAACACA
1251 ACAACAATGA ATTTCCATTT TTCTGTTTCT TTACTTCTCT CTTTATCTCT
1301 TCCTATGTTT GCCTCTTCGA CGGTGTTATT TCAGGTATCC ATCTCCAAAG
1351 AACCTTATTT TTCTCTTAAC TTTTCCTATG TATATGTATC TCTATGTTTA
1401 TGTAGTACTT GCTCAAGTAT ATAAAGAAAA GTTAGTTTCT CTAGAATCTT
1451 TGAATTCATT TGTTAGGGGT TCAATTGGGA TTCGAGTAAT AAGCAAGGCG
1501 GATGGTACAA CTCTCTCATC AACTTAGTTC CGGACTTGGC TAAAGCTGGA
1551 GTTACTCATG TTTGGTTGCC ACCATCATCT CACTCCGTTT CTCCTCAAGG
1601 TAATTTTCGG AGTGATTGTG ACCTAGTAAT CCAATGAAGT CAAAATAACC
1651 ACGGAAGATT AGAGTCTAAA TTTTAATGAA AATAGTTCAG ACAAGTTAAT
1701 GACCAACTTA TATATTAGTT CAATCCATAA AATTTGATGT AGTAGTTACA
1751 AAATGGAATT GCTTGAAGGC TTATGCCATG TTTTATGCCA GGTTATATGC
1801 CAGGAAGGTT GTATGACTAG GATGCTTCCA AGTTTGGAAA TCAGCAACAA
1851 CTGAAAACTC TTATTAAGGC TTTAACATGA CCACGGGATC AAATCGGTTG
1901 CTGATATAGT GATAAATCAT AGAACTGCTG ATAACAAAGA TAGCAGGGGA
1951 ATATACAGCA TCTTTGAAGG AGGAACATCT GATGACCGGC TTGATTGGGG
2001 TCCATCTTTC ATTTGCAGGA ACGACACACA ATATTCTGAT GGCACGGGGA
2051 ATCCAGACAC GGGTTTGGAC TTTGAACCTG CACCTGATAT CGATCATCTT
2101 AATACGAGAG TGCAGAAAGA GTTATCAGAC TGGATGAACT GGCTGAAATC
2151 TGAAATTGGA TTTGATGGTT GGCGTTTCGA TTTTGTTAGG GGATATGCAC
2201 CTTGCATTAC CAAAATTTAT ATGGGAAACA CGTCCCCGGA TTTTGCTGTT
2251 GGTGAATTGT GGAACTCTCT TGCTTATGGC CAGGACGGGA AACCGGAATA
2301 TAACCAGGAC AATCATAGAA ATGAGCTAGT TGGTTGGGTA AAAAATGCGG
2351 GGCGGGCTGT AACAGCTTTT GATTTTACAA CAAAGGGAAT TCTTCAAGCT
2401 GCAGTTCAAG AAGAGTTATG GAGATTGAAG GATCCCAATG GAAAACCTCC
2451 TGGGATGATC GGTGTTTTGC CTCGAAAAGC TGTGACTTTT ATCGATAATC
2501 ATGATACTGG ATCGACACAA AATATGTGGC CTTTCCCTTC AGACAAAGTT
2551 ATGCAAGGAT ATGCATACAT TCTTACTCAT CCAGGAATCC CATCCGTGGT
2601 AAAAAAAATA AATAAATTCT TTCTACATAT CTCATTGTTT TCTATTTTAC
2651 AAGAAATTTA TATTCTTTTC CAGGGGATTT GAGAAACTCG GCCTGTGGGA
2701 GTTTGCTCAC ATTGCCAGTC TCGTAATCCA TAAACAAACA CTCAAACTCT
2751 GAGTGTGCAC ATCTAGACAC CTCAACTCGT TTTTCACCGT GTTAATTGAA
2801 CACTTCAACT TACAAAATGA TCGTGTAGCA CCTCCAAAAA TTATGTGTCA
2851 CAATTAGCCA CGTGCGAGAT ACACGAAAAT GAGTTGGAGT AGTTAGTTGC
2901 CAAATAAAAC CAAGCTGAGG TGTCTAAATG TGCACNCTCA AAGTNGGATG
2951 TTTACTTGGC AGCTGAGGCC GAGGCCATGT TTGANTGTTA TGCTTATAGG
3001 ATATGACACA TTTGTTTCCG ATTAGCTGAG GANTTGATTA AATCCTNGTT
3051 TTNGTTNGCA GTTTNATNAC CATTNCTTTG ATNGGGGCTN CNAGGATGGA
3101 ATTNCAGCAC TAANCTCTAT TAGGAAAAGG AATAGGATTT GTGCANCAAG
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UQ406 insertion

FIGURE 10 (ii)

```
3151  CAATGTGCAA ATAATGGCTC CTGATTCTGA ATCTTTATAT ANCAATGGAT
3201  CATCACAAAA TCATTGTCAA GATTGGACCA AAACTTGATC TTGGAAATCT
3251  TATTCCACCT AATTATGAGG TGGCAACTTC TGGACAAGAC TATGCTGTAT
3301  GGGAGCAAAA GGCATAATCA TATTGTACCA CACTAAAAGG GACCATGGCC
3351  ACAATGGTTC TCATTAGTGT TAATGTTATA TGATTGAAAA TGTAATTTAT
3401  ATTGACATAA TGAAGGCCAA AAATTCAAGA AATTATAAAC AATTCAATAG
3451  TCCTTGCTCA ATTCACAATT ACATTATGAC TTCTCTATTG CAAACTAGTT
3501  TGGGTCCACA TTATTGTCTC CTAAAATTTT ACAACATTTC TTAAGGGAAC
3551  TTAATTAGTT ACAGTGAACA TATGTTGAAA TTACCCTTTA TCCCCTTACA
3601  ATTGATTTAA TAAATATTTC CCCTATCCCT TTGGTAGTTG GTTAGAGTTA
3651  TAAGTAACGT AGAGATTAGT TATAAGAGAA TTTATGTATT ATTATGCAGA
3701  TGTTTAGTTA TATCGATTTT AGTTATTTAT ATGTTGATTA TTTCACCTTC
3751  AATAATGCAT ATAAAGATGG TAAATGATTG GATTGATCGA ATTCGAATGA
3801  GTTTGAATAT GAACTAATCT TCAAATTTAA TATAAATTTT TTTTGTCAAC
3851  ATCTATAGCC AAACGGCTCC AAAACAATAA ATAATTTACA TTTATTGTAG
3901  TATTTTATTT AAAATGGGAT NTTCCTCATC CCACTTGTAC CAGTTGAAAC
3951  CCTAATAATA AGCCAATCCA ACCGTCAAAA TTACAAATTT TGAAAATTGC
4001  GCTCCTCACA GTTCTCCCCT ATTCAGATTT GATTCATTCT CTTCATTTTT
4051  TGTTTTCACA TTTTACCTCT AAATCAACAA AATTCCCTTT GTTCAAATGG  Dem ATG
4101  GTGCTAATCA CAGCCGTGAA GATCTGGAGC TTTCTGATTC CGAGTCTGAA
4151  TCCGAATATG GGTCCGAGTC TCGAACAAGG GAGGAAGAGG AAGACGAAGA
4201  TAACTACTCA GATGCTAAAA CGACGCCGTC TTCCACTGAT CGGAAACAGA
4251  GCAAAACCCC GTCTTCTTTG GATGATGTTG AAGCAAAGCT GAAAGCTTTA
4301  AAGCTTAAGT ATGGTACTCC TCATGCTAAA ACCCCCACAG CGAAAAACGC
4351  TGTTAAACTT TACCTTCATG TTGGTGGGAA CACTGCGAAT TCCAAATGGG
4401  TAGTTTCTGA TAAGGTGACA GCTTATTCGT TTGTTAAATC GGGTAGTGAG
4451  GATGGATCGG ATGATGATGA AAATGAAGAA ACTGAGGAGA ATGCTTGGTG
4501  GGTTTTGAAA ATTGGGTCGA AGGTTCGGGC TAAGATTGAT GAGAATTTGC
4551  AGCTCAAGGC ATTTAAGGAG CAGAAAAGGG TGGATTTTGT GGCGAATGGG
4601  GTTTGGGCTG TGAGATTCTT TGGGGAGGAA GAGTATAAGG CGTTCATTGA
4651  CTTATATCAG AGCTGTTTGT TTGAGAATAC TTATGGGTTT GAGGCAAATG
4701  ATGAGAATAG AGTTAAGGTG TATGGTAAAG ACTTTATGGG GTGGGCAAAT
4751  CCAGAAGCTG CGGATGATTC AATGTGGGAG GATGCTGGGG ATAGCTTCGC
4801  GAAGAGCCCT GCGTCTGAAA AGAAGACACC TTTGAGGGTT AACCATGATT
4851  TGAGGGAGGA GTTTGAGGAG GCAGCTAAAG GAGGAGCTAT TCAGAGCTTG
4901  GCATTAGGTG CGTTGGATAA TAGTTTTCTT ATAAGTGATT CTGGAATTCA
4951  GGTTGTGAGG AACTATACTC ATGGAATAAG TGGAAAAGGT GTTTGTGTCA
5001  ATTTTGATAA GGAAAGGTCT GCTGTACCTA ATTCCACTCC AAGGAAAGCT
5051  CTACTTCTAA GAGCTGAGAC TAATATGCTT CTCATGAGTC CAGTGACTGA
5101  TAGAAAGCCT CACTCTCGGG GATTACATCA GTTTGATATC GAGACTGGGA
5151  AGGTTGTTAG CGAGTGGAAG TTTGAGAAAG ATGGAACTGA TATCACGATG
5201  AGGGATATCA CTAATGATAG CAAAGGAGCT CAGATGGATC CTTCGGGGTC
5251  TACTTTCTTA GGGCTAGATG ATAACAGATT GTGTAGGTGG GATATGCGTG
5301  ATCGGCATGG GATGGTCCAG AATCTAGTTG ATGAAAGTAC TCCTGTGCTG
5351  AATTGGACTC AAGGACATCA ATTTTCGAGG GGAACTAACT TTCAGTGCTT
5401  TGCTACTACT GGTGATGGAT CAATTGTTGT TGGTTCACTT GATGGCAAGA
5451  TTAGATTGTA CTCAAGCAGT TCCATGAGAC AGGCTAAAAC TGCTTTTCCA
5501  GGCCTTGGTT CTCCTATCAC TCATGTGGAT GTTACCTATG ATGGGAAGTG
5551  GATATTGGGG ACAACTGATA CTTACTTGAT ATTGATATGC ACCTTGTTTA
5601  TCGACAAGAA TGGAACTACT AAGACTGGTT TTGCTGGTCG CATGGGAAAT
5651  AAGATTTCCG CTCCAAGATT GTTAAAGCTA AACCCTCTCG ATTCACATAT
5701  GGCTGGAGCT AACAAGTTCC GCAGTGCTCA ATTTTCATGG GTCACCGAGA
5751  ATGGGAAGCA AGAGCGCCAC CTCGTTGCTA CTGTTGGGAA GTTTAGTGTG
5801  ATCTGGAATT TTCAACAGGT GAAGGATGGT TCTCATGAGT GTTACCAGAA
5851  TCAGGTTGGG TTGAAGAGCT GCTATTGTTA CAAGATAGTC CTAAGAGACG
5901  ACTCTATTGT AGAAAGTCGT TTCATGCATG ACAAGTACGC TGTTTCTGAC
5951  TCACCTGAAG CACCACTGGC GGTAGCAACC CCCATGAAAG TCAGCTCATT
6001  CAGCATCTCT AGCAGGCGCT TACAAATTTG AACAATCATT CTGTTCATAT
6051  ACGCAACTTA TTAGATTTAT CTGTAGCAGA ATTAGTGTCT CTCACACTAA
```

FIGURE 10 (iii)

```
6101  GTAGCTTGAA AAACTGCACA TCTGCAAATC ATTTCCAGTT CAATGTATTA
6151  CTACTTTAGT TTAAAAACCT TAAAAGGCAG TCTTCCAAAT TCTAGGTATC
6201  CTCACCTGAC ATTATTATTG TTGTAATAGC TAATTGTTGC TTGCTCTAAA
6251  TCCCCGTTCA ATG
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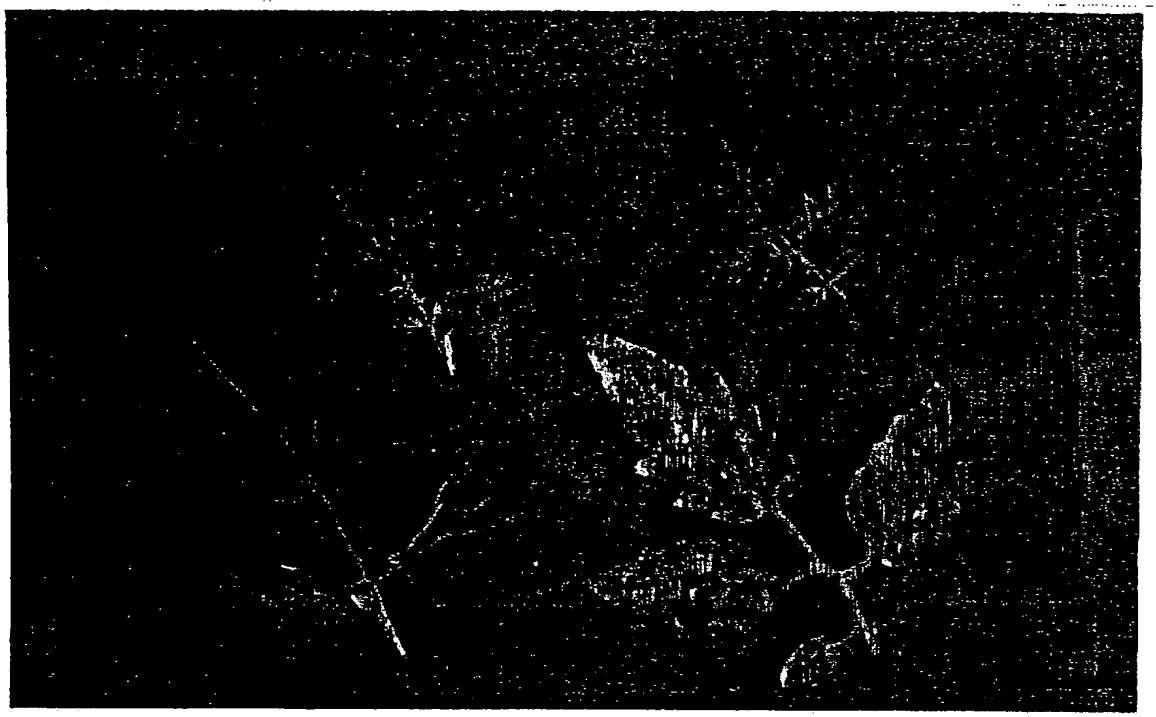

FIGURE 11